





NRPI is a Prognostic Factor and Promotes the Growth and Migration of Cells in Intrahepatic Cholangiocarcinoma

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Background: Neuropilin-1 (NRP-1) participates in cancer cell proliferation and metastasis as a multifunctional co-receptor by interacting with multiple signaling pathways. However, few studies have addressed the precise function and prognosis analysis of NRP1 in intrahepatic cholangiocarcinoma (ICC). We aimed to study the correlations between NRP1 and clinicopathological characteristics and NRP1 effect on ICC cell line functions.

Methods: NRP1 mRNA and its protein levels in human ICC tissues and cell lines were detected by IHC, qRT-PCR, and WB method. Transwell, wound healing, and CCK-8 assays were performed to verify the effects of NRP1 knockdown and overexpression on cell migration and proliferation capability.

Results: NRP1 proteins and mRNA levels increased in ICC tissues compared to those in paired adjacent non-tumor tissues. High NRP1 expression of ICC tissues was related to poor prognosis. NRP1 expression level was expected to be an independent prognosticator for overall survival and cumulative tumor recurrence, and was closely related to tumor number ($P=0.047$). Knockdown of NRP1 inhibited cell proliferation and migration capability of RBE cells in vitro, and NRP1 overexpression in 9810 cells accelerated proliferation and migration. Additionally, NRP1 may promote cell proliferation and migration in ICC via the FAK/PI3-K/AKT pathway.

Conclusion: As an oncogene, NRP1 may function as a candidate target and prognostic biomarker of value for ICC therapy.

Keywords: neuropilin-1, prognostic factor, cell growth and migration, intrahepatic cholangiocarcinoma

Introduction

Intrahepatic cholangiocarcinoma (ICC), known as the second most common liver cancer, accounts for approximately 10% of primary liver cancer. Most ICCs originate from intrahepatic bile duct epithelial cells, with strong metastatic and invasion ability and poor prognosis.^{1,3} Currently, the best way to treat ICC is through surgical resection; however, only 15% of ICC patients present with resectable ICC.^{4,5} In addition, radiotherapy and chemotherapy offer limited benefits for advanced ICC. The mortality and incidence of ICC is on the rise worldwide, and the median survival is within 3 years, seriously endangering human health.⁶ Therefore, identification of novel therapeutic molecular targets is essential for improving the prognosis of ICC.^{7,8}

NRP1 was first discovered as an adhesion molecule in the frog nervous system in 1987.⁹ It is a non-tyrosine kinase transmembrane glycoprotein located on the cell

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surface and plays a role of a co-receptor for secreted Semaphorin-3A (Sema3A). Previous studies have shown that NRP1 gene expression is widespread over a variety of cells and tissues such as endothelial cells, and the heart, liver, lung, kidney, pancreas, and skeletal muscle.¹⁰ NRP1 plays a vital role in the nervous, digestive, and immune systems, etc. through interaction with various ligands, promoting angiogenesis, neural development, cytoskeleton remodeling, inflammation, initial immune response, as well as occurrence and development of tumors.^{11,14} Noteworthy, the role of NRP1 in the occurrence and development of human malignant tumors has arisen as an interesting research topic recently.^{15,16} Increasing evidence suggests that higher expression or mutations of NRP1 are closely related to initiation, progression, and prognosis of varieties of types of malignant tumors in humans, including hepatocellular carcinoma,¹⁷ gastric cancer,¹⁸ breast cancer,^{19,20} prostatic cancer, and pancreatic cancer.^{21,22}

However, so far the expression of NRP1 and its underlying role in the occurrence, development, and prognosis of ICC have been rarely discussed to date. The important role of NRP1 in many malignant tumors has prompted us to investigate the biological effects and clinical relevance of NRP1 in human ICC. In comparison with paired adjacent non-tumor tissues, we found significantly increased expression of NRP1 protein in the tumor tissues of ICC patients. We also found that the upregulation of NRP1 was an independent prognosticator for overall survival and cumulative recurrence. In addition, *in vitro* experiment indicated that NRP1 depletion significantly inhibited the proliferation and migration capability of human ICC cell lines; however, overexpression of NRP1 resulted in opposite results. Based on these results, we conducted further experiments and found that NRP1 possibly enrolled in the net of the FAK/PI3-K/AKT pathway. To sum up, NRP1 is a valuable promoting factor in ICC and a potential target for further drug development.

Materials and Methods

Patients and Clinical Tissue Samples

Tumor tissues and paired adjacent non-tumor tissues were collected from 291 ICC patients treated at Zhongshan Hospital of Fudan University from 2010 to 2014. All patients' tissue specimens were classified as ICC through pathological examination. Fresh tissue specimens were obtained, formalin-fixed, and paraffin embedded according to a previously reported method.²³ The study was

approved by the Ethics Committee, and all the participants provided informed consent under Institutional Review Board protocols before tissue specimen collection. Patients' clinicopathological data including gender, age, tumor number, tumor size, embolus, tumor encapsulation, TNM, liver cirrhosis, NRP1, lymphonodus, capsule, serum alpha fetoprotein (AFP), CA199, and HBsAg concentration were collected before surgery for analysis.

Immunohistochemistry (IHC)

IHC was conducted on the basis of a formerly reported method with slight modifications.^{24,25} In brief, tissue microarrays were constructed using BioChip (Shanghai, China). The rabbit monoclonal antibody against human NRP1 (1:200 dilution) was purchased from Abcam (Cambridge, UK). The treated sections were placed under an optical microscope (Olympus Corp., Tokyo, Japan; magnification, $\times 200$) to observe the experimental results. For comprehensive analysis of the results, in terms of staining intensity and stained cell quantity, two specialist pathologists who lacked information about the patients separately evaluated the tissue sections. The intensity was scored as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. The frequency of positive cells was defined as follows: 0, less than 5%; 1, 5–25%; 2, 26–50%; 3, 51–75%; and 4, greater than 75%.

ICC Cell Lines and Culture

Human RBE and 9810 ICC cell lines were obtained from Shanghai Cell Bank (Shanghai, China). They were cultured under standard condition using Dulbecco's modified Eagle's medium (DMEM; Gibco, Shanghai, China) complemented with 10% fetal bovine serum (FBS; Gibco) and 1% Streptomycin-Penicillin (100 IU/mL; Gibco) at 37°C with 5% CO₂. This was followed by 1-day invasion and migration assays, 2-day qRT-PCR and WB analysis, 3-day lentivirus transfection and CCK-8 assay experiments. All operations were conducted in a biosafety cabinet after ultraviolet sterilization.

Cell Transfection

The lentiviral packaging system was composed of the vectors pGCL-puro, pHelper 1.0, and pHelper 2.0, provided by Hanyin Biotech Genechem (Shanghai, China). Three plasmids (pHelper 1.0, pHelper 2.0, and pGCL-puro or pGCL-puro-NRP1) were co-transfected into 293T cells using Lipofectamine 2000 (Invitrogen, USA). After 12 hours, the cell culture medium was replaced with fresh

medium containing 10% fetal calf serum. The lentivirus supernatant containing NRP1 cDNA was collected at 48 hours after co-transfection. Lentivirus supernatant was transfected into 9810 cells with 8 µg/mL polybrene (Sigma-Aldrich, ST. Louis, MO, USA) to obtain a stable cell line, named 9810-OE, the 9810-NC was used as a control. A set of shRNA (short hairpin RNA) lentiviral vectors differed in NRP1-targeting sequences (Supplementary [Table S1](#)) and pLKO.1-puro empty vector with green fluorescent protein (GFP) were purchased from Genechem (Shanghai, China). The lentiviral packaging system was the same as mentioned above. Lentivirus supernatant was transfected into RBE cells with 8 µg/mL polybrene (Sigma-Aldrich) to obtain a stable cell line, named RBE-shNRP1. The RBE-NC was used as a control.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

In our study, qRT-PCR was performed according to a previously reported method.²² NRP1 and GAPDH (inner control) primers were synthesized and purchased from Sangon Biotech (Shanghai, China): NRP1 sense, 5'-ATCACGTGCAGCTCAAGTGG-3', and antisense, 5'-TCATGCAGTGGCAGAGTTC-3'; GAPDH sense, 5'-GGTATGACAACGAATTTGGC-3', and antisense, 5'-GAGCACAGGGTACTTTATTG-3'. The experiments were repeated in triplicate.

WB Analysis

In this study, WB was performed according to a previously reported method with slight modification.^{24,25} Briefly, RIPA lysis buffer containing protease and phosphatase inhibitors for extracting total protein from tissues and cells was purchased from Sigma-Aldrich. Anti-human NRP1 antibody (1:2000 dilution) was purchased from Abcam. Finally, relative proteins were quantified using Image-pro plus 6.0 (Media Cybernetics, USA).

Cell Proliferation, Invasion, and Migration Assays

To explore cell proliferation activity, we performed CCK-8, assay experiments in six replicates. To evaluate cell migration capability, we performed transwell and wound healing assays separately, and the experiments were performed in triplicate. CCK-8, transwell, and wound healing assays were carried out according to a previously reported

method.²⁴ We blocked proliferation in the wound healing assays with 2% serum concentration. The variation in migration capability of cells can be determined by observing and photographing using a light microscope (Olympus Corp.; magnification, ×100).

Statistical Analysis

Data were analyzed using SPSS v.21.0 software (IBM Corp., Chicago, IL, USA). The correlation between NRP1 expression and clinicopathological characteristics were analyzed using Fisher's exact test or Pearson chi-squared test. We used multi-factor or one-way ANOVA to analyze the significance of differences among groups. Overall survival and cumulative recurrence were described using Kaplan-Meier plots (Log rank tests). The data for cell functional assays, described as means±standard deviation, were evaluated by Mann-Whitney *U*-test. $P < 0.05$ was regarded as representing a statistically significant difference.

Results

Expression of NRP1 in ICC Tissues and Its Correlation with Prognosis and Clinicopathological Characteristics

[Figure 1](#) shows the immunohistochemical expression of NRP1 in ICC tissues and adjacent non-tumor tissues. Light microscopy showed that the expression levels of NRP1 protein between ICC and adjacent tissues were different. In total, 188 samples (64.60%) showed high NRP1 expression, while 103 samples (35.40%) had low NRP1 expression. The overall survival and cumulative tumor recurrence in ICC patients were evaluated using Kaplan-Meier analysis, thus revealing that high NRP1 expression was significantly linked with decreased overall survival and high risk of cumulative tumor recurrence ([Figure 2](#)). Further, the high NRP1 expression always indicates a poor prognosis. The results of correlation between NRP1 expression and clinicopathological characteristics ([Table 1](#)) showed that NRP1 upregulation was significantly associated with tumor number ($P=0.047$). No significant difference showed in age, gender, tumor size, AFP expression, CA199 expression, and TNM of NRP1 expression in patients with ICC ($P > 0.05$). Furthermore, univariate and multivariate Cox regression analyses ([Table 2](#)) demonstrated that NRP1 is an independent prognosticator of overall survival ($P=0.000$) and cumulative tumor recurrence ($P=0.021$).

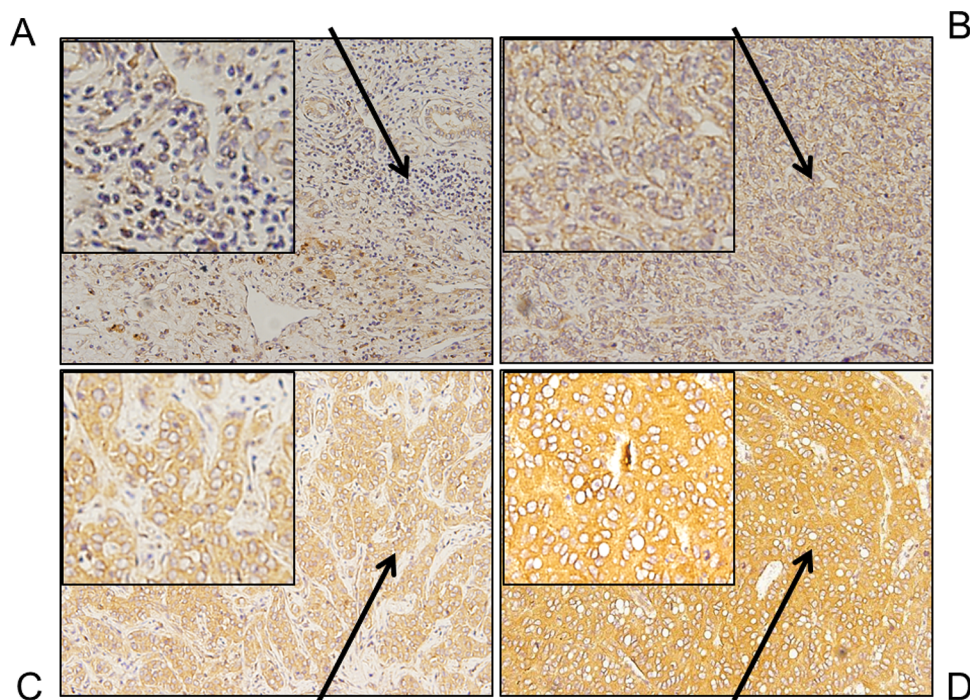


Figure 1 NRPI expression detection in both ICC tissues and adjacent non-tumor tissues using IHC analysis; Magnification, $\times 200$. **(A)** The staining in adjacent non-tumor tissue is weak, indicating that the expression level of NRPI is low. **(B)** Weak staining in tumor tissues, indicating that the expression level of NRPI is low. **(C)** Moderate staining in tumor tissues, indicating the general expression level of NRPI. **(D)** Strong staining in tumor tissues, indicating that the expression level of NRPI is high.

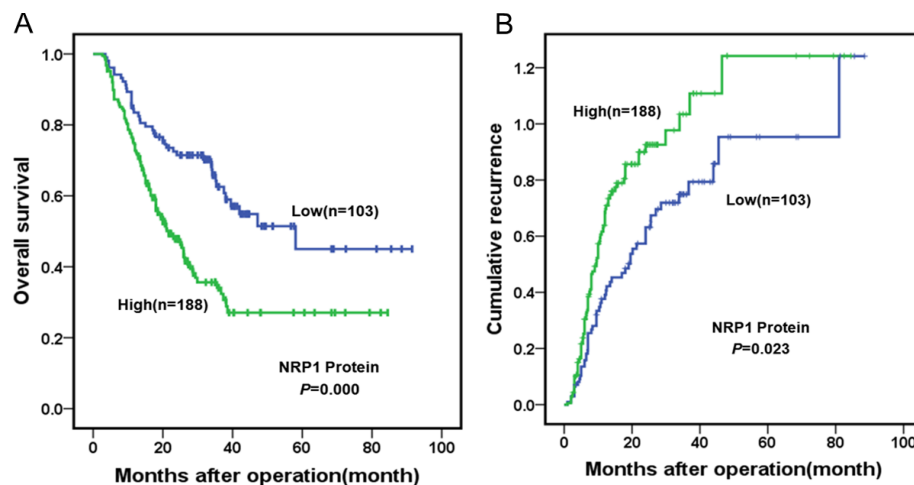


Figure 2 IHC staining level showing overall survival and cumulative tumor recurrence in ICC patients, evaluated using Kaplan–Meier analysis. **(A)** The overall survival of 103 ICC patients (35.40%) with low NRPI expression was higher than that of 188 ICC patients (64.60%) with high NRPI expression ($P < 0.001$). **(B)** The cumulative recurrence of 103 ICC patients (35.40%) with low NRPI expression was lower than that of 188 ICC patients (64.60%) with high NRPI expression ($P = 0.023$).

Expression of NRPI is Upregulated in Human ICCs

The expression of NRPI was markedly increased in ICC tissues than that in adjacent non-tumor tissues at protein and mRNA levels. Specifically, NRPI mRNA expression in 55 ICC tissues and their paired adjacent tissues were evaluated by qRT-PCR. Among the 55 groups, cancer

tissues had higher levels of NRPI mRNA than those in adjacent tissues in 33 pairs (60.00%) (Figure 3A). At the protein level, cancer tissues had higher expression levels of NRPI proteins than those in adjacent tissues in eight out of 15 pairs (53.33%) (Figure 3D and E). Also, we assessed NRPI protein expression levels in ICC cell lines by Western blot (WB), which revealed that the highest

Table I Correlations Between the Factors and Clinicopathological Characteristics in ICC (n=291)

Clinicopathological Indexes		NRPI		
		Low	High	P
Age (years)	≤50	56	96	0.625
	>50	47	92	
Sex	Female	44	73	0.534
	Male	59	115	
AFP (ng/mL)	≤20	89	170	0.529
	>20	11	16	
CA199 (ng/mL)	≤30	46	94	0.611
	>30	48	85	
Tumor size (cm)	≤5	41	90	0.218
	>5	62	98	
Tumor number	Single	83	136	0.047
	Multiple	20	52	
Embolus	No	89	159	0.732
	Yes	14	29	
Capsule	No	11	25	0.580
	Yes	92	163	
TNM	I/II	84	140	0.192
	III/IV	19	48	
Lymphonodus	No	88	152	0.335
	Yes	15	36	
Liver cirrhosis	No	79	133	0.420
	Yes	24	55	

NRPI expression level was found in the RBE cell line, whereas the lowest was observed in the 9810 cell line (Figure 3B and C).

NRPI Depletion Impairs Proliferation, Invasion, and Migration of RBE Cell Line in vitro

To explore the role of NRPI in ICC cell lines based on the above experimental results, we knocked out NRPI from the RBE cell line having high expression of NRPI. As shown in Figure 4, the normal control group was defined as the RBE-NC group and the NRPI depletion group was defined as the RBE-shNRPI group. After silencing, the expression of NRPI mRNA level in the RBE-shNRPI group decreased significantly (Figure 4A). Figure 4C presents the validation of transfection efficiency at the protein level. Figure 4B shows CCK-8, Figure 4D and E show transwell, and Figure 4F and G show wound healing

assay results, respectively. CCK-8 assay was used to analyze cell proliferation. Transwell and wound healing assays were used to evaluate cell invasion and migration capability. The results revealed that eliminating NRPI impairs proliferation, invasion, and migration in RBE cells.

NRPI Overexpression Promotes Proliferation, Invasion, and Migration of 9810 Cell Line in vitro

To further prove the role of NRPI in ICC cell lines, we overexpressed NRPI in the 9810 cell line, which has the lowest expression of NRPI. In Figure 5, the normal control group was defined as the 9810-NC group and the NRPI overexpression group was defined as the 9810-OE-NRPI group. After overexpression, the expression of NRPI mRNA in the 9810-OE-NRPI group increased significantly (Figure 5A). Figure 5C shows the validation of

Table 2 Univariate and Multivariate Analyses of Prognostic Factors in ICC (n=291)

Variable	Cumulative Recurrence		Overall Survival	
	HR (95% CI)	P	HR (95% CI)	P
Univariate analysis				
Age, years (≤ 50 vs > 50)	0.872 (0.639–1.189)	0.387	1.208 (0.877–1.662)	0.247
Sex (female vs male)	1.051 (0.768–1.440)	0.756	1.204 (0.864–1.677)	0.273
AFP, ng/mL (≤ 20 vs > 20)	0.948 (0.548–1.641)	0.850	0.966 (0.557–1.676)	0.902
Tumor size, cm (≤ 5 vs > 5)	1.334 (0.977–1.822)	0.070	1.507 (1.086–2.091)	0.014
Tumor number (single vs multiple)	1.890 (1.353–2.641)	0.000	1.766 (1.250–2.495)	0.001
Embolus (no vs yes)	1.586 (1.072–2.346)	0.021	1.202 (0.776–1.861)	0.409
Tumor encapsulation (complete vs none)	1.505 (0.898–2.524)	0.121	1.357 (0.796–2.313)	0.262
TNM (I/II vs III/IV)	2.009 (1.424–2.834)	0.000	2.480 (1.764–3.486)	0.000
NRPI (low vs high)	0.688 (0.496–0.955)	0.025	0.452 (0.314–0.652)	0.000
Liver cirrhosis (no vs yes)	1.256 (0.896–1.760)	0.186	1.084 (0.757–1.552)	0.661
Lymphonodus (no vs yes)	2.529 (1.749–3.657)	0.000	2.946 (2.046–4.242)	0.000
CA199, ng/mL (≤ 30 vs > 30)	1.280 (0.934–1.754)	0.125	1.586 (1.131–2.222)	0.007
HBsAg (no vs yes)	0.895 (0.652–1.228)	0.491	0.712 (0.509–0.996)	0.047
Multivariate analysis				
Tumor size, cm (≤ 5 vs > 5)			1.564 (1.084–2.255)	0.017
Tumor number (single vs multiple)	1.761 (1.251–2.479)	0.001	1.783 (1.224–2.597)	0.003
TNM (I/II vs III/IV)	1.078 (0.507–2.291)	0.845	1.133 (0.565–2.273)	0.725
Lymphonodus (no vs yes)	2.024 (0.921–4.448)	0.079	2.298 (1.120–4.716)	0.023
CA199, ng/mL (≤ 30 vs > 30)			1.483 (1.052–2.091)	0.024
HBsAg (no vs yes)			0.708 (0.493–1.016)	0.061
NRPI (low vs high)	0.675 (0.483–0.942)	0.021	0.461 (0.312–0.683)	0.000
Embolus (no vs yes)	1.429 (0.955–2.138)	0.083		

transfection efficiency at the protein level. Figure 5B shows the results of CCK-8, Figure 5D and E show the results of transwell, and Figure 5F and G show wound healing assays. The results indicate that overexpression of NRPI promoted proliferation, migration, and invasion of 9810 cells.

p-FAK/p-PI3-K/p-AKT Pathway Could Be One of the Possible Mechanisms Behind the Effects of NRPI on Cell Function

To ascertain the signaling pathways that contribute to NRPI-related ICC transformation, we used WB to analyze the expression of key proteins including NK- κ B, p-FAK, p-PI3-K, Paxillin, and p-AKT in common pathways, and the relative proteins were normalized to β -actin levels. As shown in Figure 6, downregulation of NRPI in RBE cells by shRNA significantly reduced p-FAK, p-PI3-K, and p-AKT pathway activities compared to those in RBE-NC cells. Conversely, FAK, PI3-K, and AKT were clearly activated in 9810-OE cells relative to 9810-NC cells (Figure 6). The above results indicate that NRPI depletion

inhibits the activation of the p-FAK/p-PI3-K/p-AKT pathway, while high expression of NRPI promotes the activation of the p-FAK/p-PI3-K/p-AKT pathway.

Discussion

ICC is a highly invasive malignant tumor associated with an extremely poor prognosis, and radical resection is the only effective treatment presently. However, a vast majority of patients have different sites of invasion and metastasis at the time of identification. Therefore, early diagnosis and prevention of ICC invasion and metastasis are key steps to enhance the efficacy of treatment.^{26,28} Several reports have shown that mutations or elevated expression of NRPI are correlated with many human malignancies.^{17,18,20,22,29} However, the expression of NRPI and its possible role in the occurrence, development, and prognosis of ICC have been rarely discussed. The relationship between NRPI expression and clinicopathology in ICC remains unclear.

In our research, based on a large number of organization samples from 291 ICC patients, we preliminarily studied the expression of NRPI in ICC tissues and its

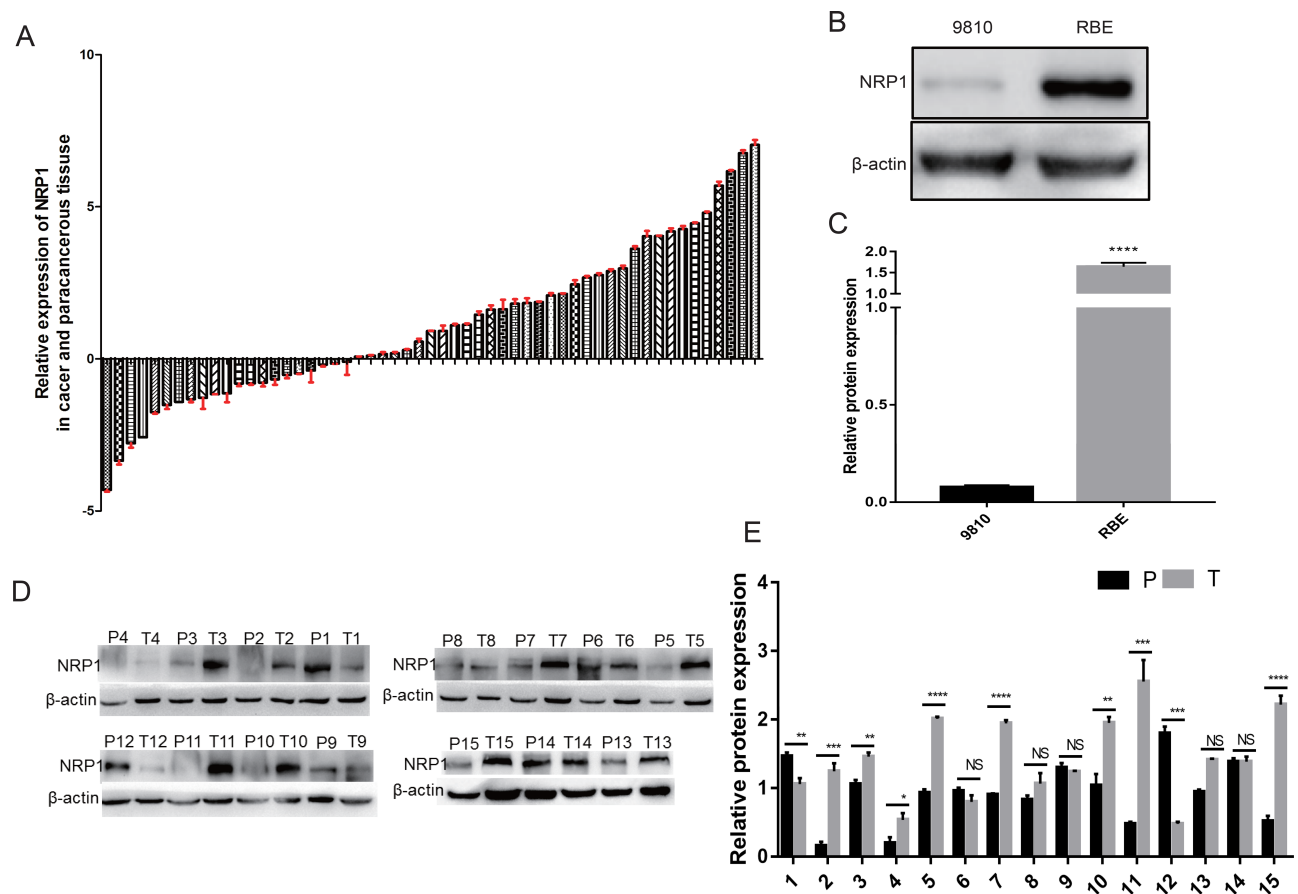


Figure 3 Expression difference of NRP1 mRNAs and its proteins between ICC tissues and adjacent non-tumor tissues. **(A)** The expression level of NRP1 mRNAs in 55 paired groups was detected using qRT-PCR (T, tumor tissue; P, adjacent non-tumor tissue). **(B and C)** The expression level of NRP1 proteins in ICC cell lines. **(D and E)** Relative NRP1 expression in 15 paired groups was analyzed using WB. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, and **** $P<0.0001$.

correlation with prognosis and clinicopathological characteristics, and we found that NRP1 is associated with poor prognosis and may be a cancer promoting factor. Although a previous study³⁰ reported that NRP1 is increased in human cholangiocarcinoma tissue, they chose NRP1 based on the previous gastric cancer study, and only pay attention to the effect of NRP1 silencing on cell proliferation. More perfectly, we focused on the effect of NRP1 silencing and overexpressing on proliferation, invasion, and migration of ICC cell lines. Moreover, this paper only measured the expression of NRP1 in five patients with cholangiocarcinoma, while our research measured the expression of NRP1 in 55 patients with carcinoma and paired normal tissues.

The important role of NRP1 in many malignant tumors has prompted us to investigate the clinical relevance, prognostic evaluation, and biological effects of NRP1 in human ICC. In our study, we demonstrated the relationship between high NRP1 expression and decreased overall survival and high risk of cumulative recurrence in ICC

patients. Interestingly, although 35.40% of ICC patients had lower NRP1 levels in tumors than those in the adjacent non-tumor tissues, ICC generally showed an upregulation of NRP1, and high NRP1 expression indicated poor prognosis in all ICC patients. This suggested that NRP1 upregulation might occur in parallel contributing to the oncogenic process. It is worth mentioning that the NRP1 expression was significantly correlated with tumor number, and was an independent predictor of overall survival and cumulative recurrence. Patients with low NRP1 expression were 0.461 and 0.675 times less likely to suffer from tumor recurrence and death, respectively, than those with high NRP1 expression.

However, the specific effects of NRP1 on cell function are still unclear. Accordingly, further verification was essential. Based on the above results showing that the highest NRP1 expression was found in the RBE cell line and the lowest in 9810 cells, we overexpressed the NRP1 gene in the 9810 cell line and silenced it in the RBE cell line. The cell function experiments showed that

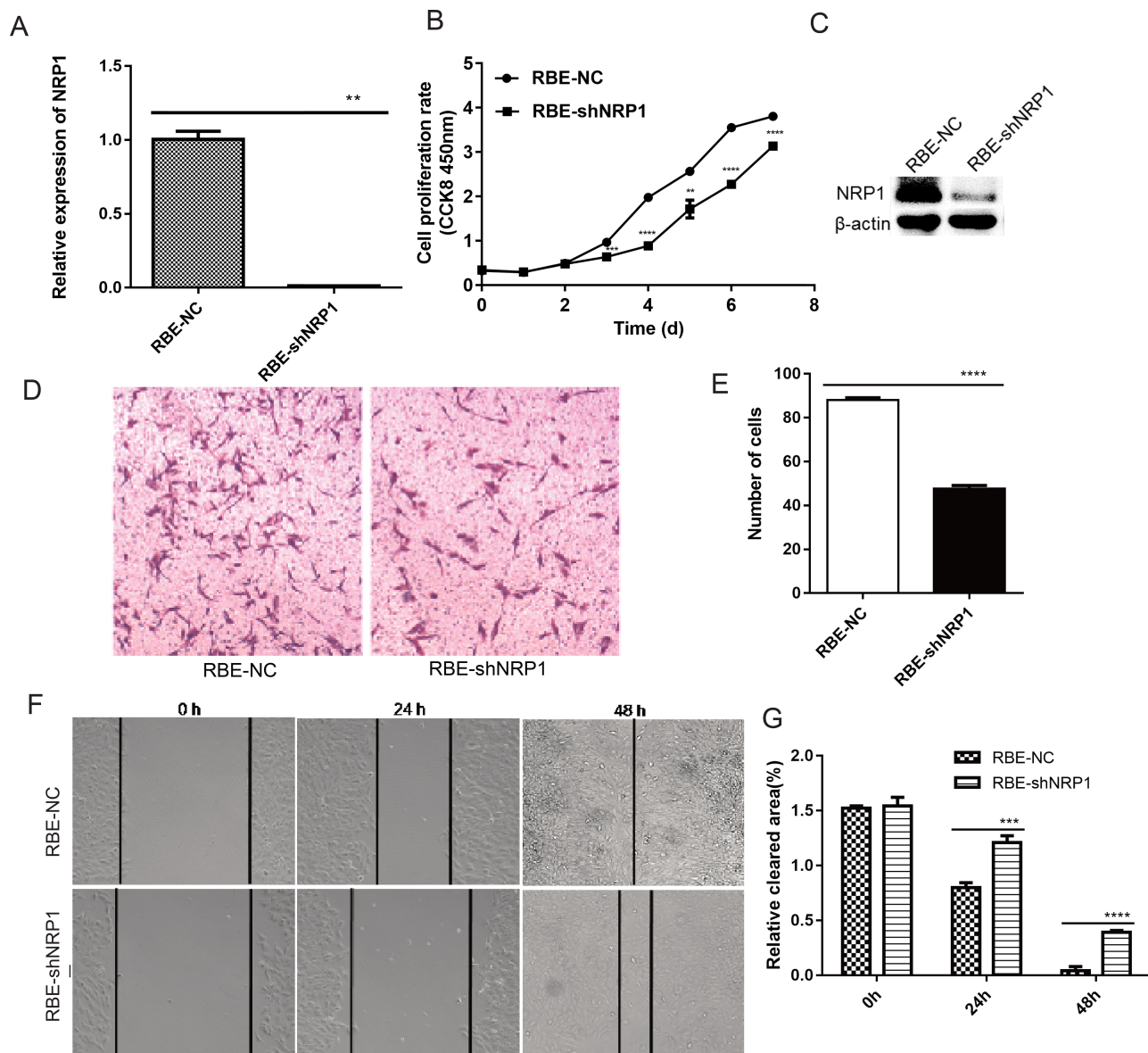


Figure 4 Cellular proliferation and migration comparison after NRP1 knockdown in the RBE cell line. **(A)** NRP1 expression levels in stably transfected RBE cells was confirmed by qRT-PCR. **(B)** CCK-8 assay confirmed that the proliferation ability of RBE-NC cells was better than that of RBE-shNRP1 cells. **(C)** Western blotting confirmed NRP1 expression levels in stably transfected ICC cells. **(D and E)** Transwell and **(F and G)** Wound healing assays of RBE-shNRP1 cells showed a considerable inhibitory effect on cell migration. Magnification, $\times 100$. ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

overexpression of NRP1 increased the proliferation, invasion, and migration abilities of the 9180 cell line. Conversely, knockdown of NRP1 in the RBE cell line impaired the proliferation and migration abilities of cells. These findings are in agreement with the findings of Zhu et al,³⁰ who showed that NRP1 depletion inhibits cell migration, proliferation, and angiogenesis.

Previous research has shown that focal adhesion kinase (FAK) is a cytoplasmic non-receptor tyrosine kinase and plays a pivotal role in cell migration, adhesion, and infiltration.³¹ FAK is involved in many cancer cells

signaling pathways, which can transmit extracellular signals to cells via integrins and growth factor receptors. It is also associated with the PI3-K/AKT signaling pathway and can promote cytoskeletal reconstruction, formation, and renewal of adhesion plaques, and the expression of matrix metalloproteinase on the cell surface, thus promoting the migration and infiltration of cancer cells.³² Numerous research has shown that the FAK/PI3-K/AKT signaling pathway is intimately connected to the occurrence and development of ICC as well as the effectiveness of chemotherapy on ICC.^{33,34} In this study, we further

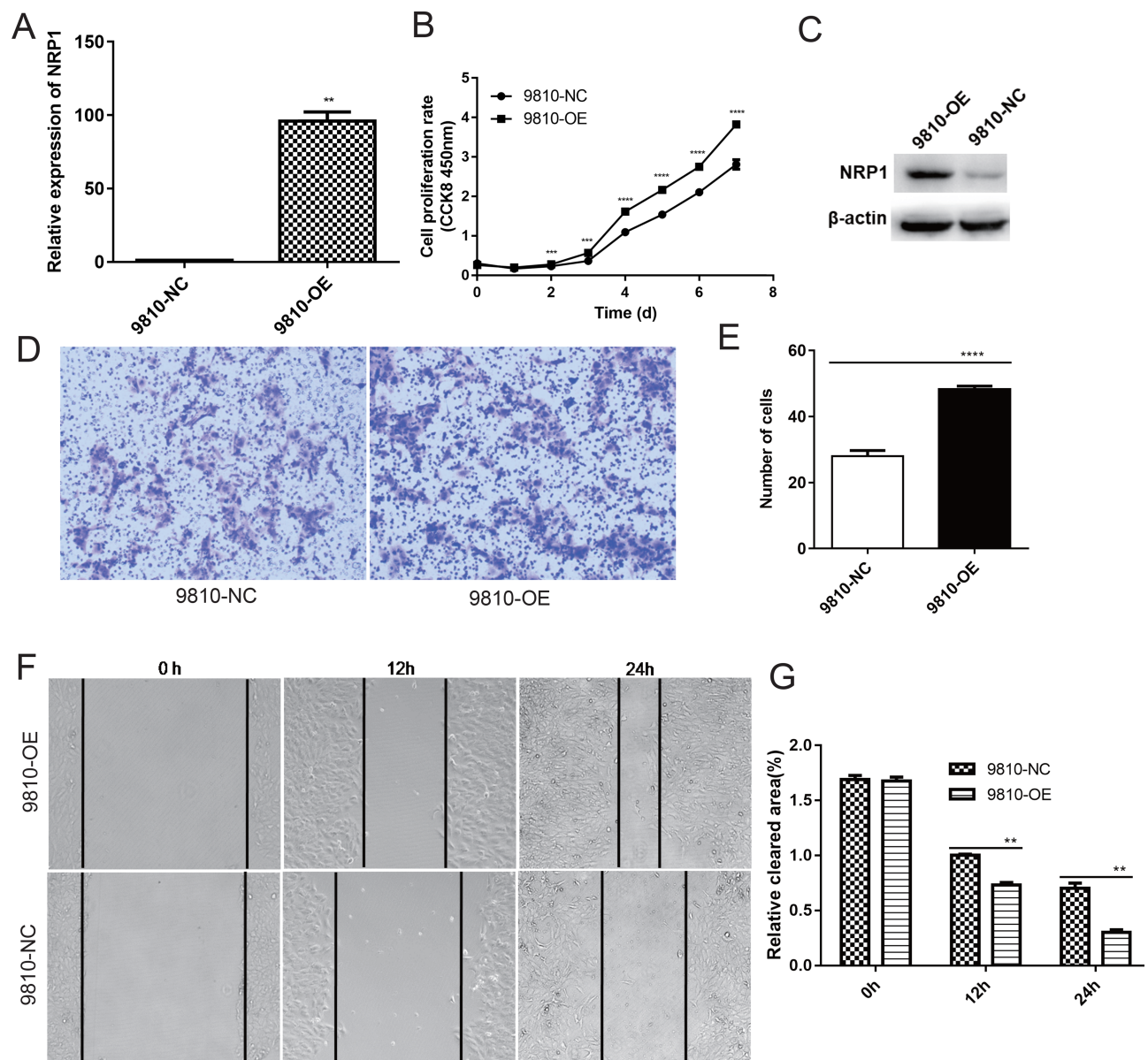


Figure 5 Cellular proliferation and migration comparison after NRP1 overexpression in the 9180 cell line. **(A)** qRT-PCR confirmed NRP1 expression levels in stably transfected ICC cells. **(B)** CCK-8 experiment confirmed that the proliferation ability of 9810-OE-NRP1 cells was better than that of 9810-NC cells. **(C)** Western blotting confirmed NRP1 expression levels in stably transfected ICC cells. **(D and E)** Transwell and **(F and G)** wound healing assays of 9810-OE-NRP1 cells showed a considerable inhibitory effect on cell migration capability. Magnification, $\times 100$. ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

elucidated the signal transduction pathway and molecular mechanism of NRP1 in ICC. Based on the above findings, we speculated and verified that NRP1 may promote the proliferation, and migration of ICC cells via the p-FAK/p-PI3-K/p-AKT pathway, which provides a new candidate target for inhibiting ICC proliferation and migration. Similarly, tolerance of ICC to conventional chemotherapy drugs is a major challenge during ICC treatment, and the mechanism of the tolerance may be related to the activation of the PI3-K/AKT signaling pathway.³⁵ Reducing the expression of NRP1 and inhibiting PI3-K/AKT signaling

may solve the problem of ICC tolerance to conventional chemotherapeutic drugs.

It is worth mentioning that previous studies have shown that NRP-1 can result in increased phosphorylation of FAK through the VEGF/VEGFR2 pathway, which is essential cancer metastasis.^{36,37} NRP-1 can also activate the HGF/c-Met pathway, leading to AKT activation and downregulation of p27.^{37,39} In addition to the PI3-K/AKT pathway, NRP-1 can promote cholangiocarcinoma cells proliferation and metastasis through the MEK/ERK pathway.³⁰ Although the above signaling pathways were

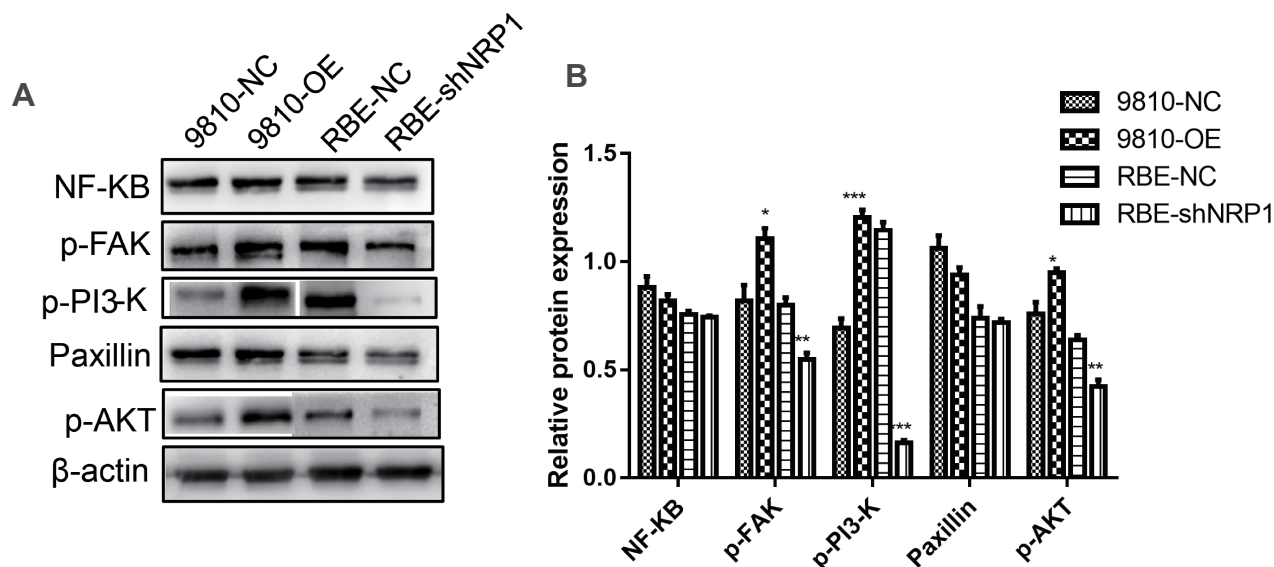


Figure 6 The expression level of NF-κB, p-FAK, p-PI3-K, Paxillin, and p-AKT. (A) The proteins in four types of ICC cells were detected using WB. (B) The relative proteins and the density of each band were normalized to β-actin levels. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

not investigated in this study, the crosstalk among the signaling pathways may promote the proliferation, invasion, and metastasis of ICC cells.

In conclusion, our study demonstrated that NRP1 is a valuable tumor-promoting and prognostic factor. The enigma behind NRP1 upregulation needs further studies to deeply investigate, which could be useful in the development of more effective targeted strategies to prevent ICC progression. To understand the function of NRP1 in ICC more comprehensively, more research will be needed, such as in vivo animal experiments and upstream and downstream signaling pathway experiments.

Data Sharing Statement

All data generated or analyzed during the present study are included in this published article.

Ethics Approval and Consent

All experiments conducted in the present study were approved by the Ethics Committee of the First Hospital of Lanzhou University (Gansu, China), and written informed consent was obtained.

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that they have no competing interests.

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